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Oral Toxicity of Carbon Tetrachloride: Acute Subacute, and Subchronic Studies in Rats

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16. ABSTRACT This investigation was conducted to characterize the acute, subacute and subchronic toxic potency of ingested carbon tetrachloride (CCl_4). In the first acute and subchronic toxicity study, male Sprague-Dawley rats of 300-350 g were gavaged with 0, 20, 40 or 80 mg CCl_4/kg once dialy for 5 consecutive days, rested for 2 days, and dosed once daily for 4 additional days. Rats of 200-250 g were gavaged with 0, 20, 80 or 160 mg CCl_4/kg according to the same dosage regimen in the second acute and subacute study. In the first and second studies one group of rats at each dosage-level was sacrificed for clinical chemistry and histopathological evaluation at 24 hr, 4 days and 11 days after initiation of dosing. Single 20 and 40 mg/kg doses had no apparent toxic effect at 24 hr, though 80 mg/kg caused mild hepatic centrilobular vacuolization and significant increases in some serum enzyme levels. In general, there was progressively severe hepatic injury at each dosage-level over the 11-day period. CCl_4 was more hepatotoxic to the 200-250 g rats than to the 300-350 g rats. In the subchronic study, rats initially 200-250 g were gavaged 5 times weekly for 12 weeks with 0, 1, 10 or 33 mg CCl_4/kg. Body weight and clinical chemistry indices wre monitored during the 12 weeks of dosing and 2 weeks after cessation of dosing. One mg/kg had no apparent adverse effect. Ten mg/kg produced slight, but statistically significant increases in sorbitol dehydrogenase activity and mild hepatic centrilobular vacuolization. Thirty-three mg/kg caused marrked hepatotoxicity. Serum enzyme

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Oral Toxicity of Carbon Tetrachloride: Acute, Subacute, and Subchronic Studies in Rats[1]

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Oral Toxicity of Carbon Tetrachloride: Acute, Subacute, and Subchronic Studies in Rats. BRUCKNER, J. V., MACKENZIE, W. F., MURALIDHARA, S., LUTHRA, R., KYLE, G. M., AND ACOSTA, D. (1986). *Fundam. Appl. Toxicol.* **6**, 16–34. This investigation was conducted to characterize the acute, subacute, and subchronic toxic potency of ingested carbon tetrachloride (CCl_4). In the first acute and subacute toxicity study, male Sprague–Dawley rats of 300–350 g were gavaged with 0, 20, 40, or 80 mg CCl_4/kg once daily for 5 consecutive days, rested for 2 days, and dosed once daily for 4 additional days. Rats of 200–250 g were gavaged with 0, 20, 80, or 160 mg CCl_4/kg according to the same dosage regimen in the second acute and subacute study. In the first and second studies one group of rats at each dosage level was sacrificed for clinical chemistry and histopathological evaluation at 24 hr, 4 days, and 11 days after initiation of dosing. Single 20- and 40-mg/kg doses had no apparent toxic effect at 24 hr, although 80 mg/kg caused mild hepatic centrilobular vacuolization and significant increases in some serum enzyme levels. In general, there was progressively severe hepatic injury at each dosage level over the 11-day period. CCl_4 was more hepatotoxic to the 200–250-g rats than to the 300–350-g rats. In the subchronic study, rats initially 200–250 g were gavaged 5 times weekly for 12 weeks with 0, 1, 10, or 33 mg CCl_4/kg. Body weight and clinical chemistry indices were monitored during the 12 weeks of dosing and 2 weeks after cessation of dosing. A dose of 1 mg/kg had no apparent adverse effect; 10 mg/kg produced slight, but statistically significant increases in sorbitol dehydrogenase activity and mild hepatic centrilobular vacuolization; 33 mg/kg caused marked hepatotoxicity. Serum enzyme levels remained elevated during the 12-week dosing period, but returned toward normal within 13 days of cessation of CCl_4 exposure. Microscopic examination of livers of the 33-mg/kg rats revealed cirrhosis, characterized by bile duct proliferation, fibrosis, lobular distortion, parenchymal regeneration, hyperplastic nodules, and single-cell necrosis. The fibrosis was not reversed within the 13-day recovery period. © 1986 Society of Toxicology.

Carbon tetrachloride (CCl_4) has enjoyed widespread use as a cleaning agent, solvent, grain fumigant, and intermediate in the synthesis of chlorofluorocarbons. However, due to the recognized toxicity of CCl_4 and the decreased utilization of fluorocarbons, production of CCl_4 has declined steadily since 1974. Nevertheless, approximately 710 million lb of the chemical were manufactured in the United States in 1980 (USITC, 1981). Thus, humans may be exposed to CCl_4 in some occupational settings and in the general environment. Levels of CCl_4 in the air are usually quite low (Singh *et al.*, 1976), though relatively high concentrations (i.e., 10–100 times normal) have been measured in the atmosphere of some urban areas (Lillian *et al.*, 1975). Food is another potential source of CCl_4 exposure, though lev-

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els measured in foods are generally in the ppb range (McConnell *et al.*, 1975–76). Much higher residue levels have been reported in grains fumigated with CCl_4 and in foods prepared from such grain (Jagielski *et al.*, 1978).

Drinking water is of particular concern at present as a source of human exposure to CCl_4 and other organic chemicals. CCl_4 was found in relatively low concentrations (i.e., ≤ 6 μg/liter) in the drinking water of a number of cities surveyed in two nationwide studies (Symons *et al.*, 1975; NOMS, 1977). Substantially higher concentrations, however, have been detected in groundwater and in surface water following CCl_4 spills. Recently 22,000 μg/liter were found in water taken from a well drilled in the proximity of a hazardous waste disposal site in Louisiana (U. S., 1983). Concentrations of up to 340 and 100 μg/liter were measured in raw and treated river water, respectively, following discharge of an estimated 70 tons of CCl_4 by a chemical manufacturer into the Kanawha River in West Virginia (Marx, 1977).

It is necessary to establish guidelines in order to protect against adverse health effects from ingestion of CCl_4-contaminated food and water. Ideally, reliable information on CCl_4 toxicity in humans would be used to derive exposure guidelines. Many cases of human poisonings from CCl_4 ingestion are reported and compiled by NIOSH (1975) and U.S. EPA (1983). The liver and kidneys are primarily affected in humans. The accounts of human poisoning are useful in defining target organ toxicity and ranges of toxic doses, but their utility in risk assessment is limited by many uncontrolled variables, incomplete accounts, and apparent differences in susceptibility to poisoning.

In the absence of reliable human data, it is necessary to use data from animal experimentation to establish dosage levels which can be utilized to calculate ingestion guidelines. The no-observed-adverse-effect level (NOAEL) and the lowest-observed-adverse-effect level (LOAEL) are two key values which should be derived from appropriately designed and controlled animal studies. Unfortunately, NOAELs and/or LOAELs for acute, subacute, and chronic CCl_4 ingestion are generally not available.

Although CCl_4 is one of the most widely studied of all toxic chemicals, there are very few oral dosing studies which have yielded definitive dose–response relationships for noncarcinogenic effects of the chemical. Although Klaassen and Plaa (1966, 1967), Drotman and Lawhorn (1978), and others describe dose–response relationships for acute CCl_4 toxicity in animals dosed by ip injection, such information is of limited utility due to the uncertainties of route-to-route extrapolations. Korsrud *et al.* (1972) do report one dose–response study in which male rats are given CCl_4 as a single oral dose.

Although there have been a number of subchronic and chronic toxicity studies of CCl_4, none have suitably defined oral dose–response relationships. Smyth *et al.* (1936), Adams *et al.* (1952), and Prendergast *et al.* (1967) have evaluated the responses of several species to different concentrations of CCl_4 vapor in long-term experiments, but the validity of extrapolation from inhalation to oral exposure remains to be determined. Mice and rats were given CCl_4 orally 5 times weekly for up to 78 weeks in a NCI (1976) cancer bioassay. Unfortunately, it has not been possible to discern a NOAEL or a LOAEL from the study report. Alumot *et al.* (1976) have determined such values for increased triglycerides and total lipids in livers of rats fed diets containing CCl_4 for 5 to 6 weeks. Unfortunately, the results of a 2-year CCl_4 feeding study by the same investigators are of limited utility due to chronic respiratory disease and poor survival of the rats.

In light of the paucity of data applicable to risk assessment of the noncarcinogenic effects of ingested CCl_4, it was decided to undertake the present study. The overall objective of the study was to characterize the acute, subacute, and subchronic toxic potency of ingested CCl_4 in rats. Dose–response relationships for selected hepatotoxicity indices were evaluated

at intervals for up to 3 months, with particular emphasis placed on delineation of NOAELs and LOAELs. Additional aims of the subchronic study were to: (a) determine whether injury was progressive or reversible upon repeated CCl_4 ingestion; (b) ascertain whether long-term ingestion of CCl_4 resulted in toxic manifestations not seen on short-term exposure; (c) assess the ability of subjects to recover from liver damage caused by long-term CCl_4 ingestion.

METHODS

Animals. Adult, male Sprague–Dawley rats from TIMCO Breeding Laboratories (Houston, Tex.) were utilized. The animals were acclimated to reverse light–dark conditions for 2 weeks prior to use, with light from 2100 to 0900 hr and darkness from 0900 to 2100 hr. Each rat was assigned an identification number which was marked on its tail. The animals were housed in groups of five or six in stainless-steel cages. Ralston Purina Formulab chow and tap water were available *ad libitum* throughout the acclimation and testing periods.

Chemical preparation and dosing. Analytical-grade CCl_4 was obtained from Matheson, Coleman and Bell (Norwood, Ohio). All other chemicals and biologicals were purchased from Sigma Chemical Company (St. Louis, Mo.). Appropriate amounts of Mazola corn oil from Best Foods/CPC International (Englewood Cliffs, N.J.) were used as diluent, such that required doses of CCl_4 were given orally in a total volume of 1 ml/animal. Control animals received 1 ml of corn oil. Each animal was dosed by gavage using a curved, ball-tipped intubation needle affixed to a 1-ml syringe. Dosings were carried out between 1000 and 1100 hr, or approximately 1 to 2 hr into the rats' active/dark cycle.

Safety precautions. The studies were conducted in a biohazards facility in order to avoid CCl_4 contamination of laboratory personnel or surrounding areas. Preparation of test solutions, dosing of animals, blood sampling, and necropsies were performed under a 100% exhaust hood. Dosed animals were transferred to and maintained within animal isolation modules. Animal remains and other CCl_4-contaminated materials were sealed in double plastic bags, placed into appropriate barrels, and disposed of by a licensed disposal firm.

First acute and subacute study protocol. Rats weighing 300 to 350 g were randomly divided into groups of five animals each. Four dosage levels of CCl_4 were utilized: 0, 20, 40, and 80 mg/kg po. The animals were dosed once daily for 5 consecutive days, allowed 2 days without dosing, and dosed once daily for 4 additional days. One group of rats at each dosage level was sacrificed at the following times after the initiation of dosing: 24 hr, 4 days, and 11 days.

Second acute and subacute study protocol. Rats weighing 200 to 250 g were randomly divided into groups of five animals each. Four dosage levels of CCl_4 were utilized: 0, 20, 80, and 160 mg/kg po. The animals were dosed and sacrificed as in the first acute and subacute study.

Subchronic study protocol. Rats weighing 200 to 250 g were randomly divided into groups of 15 to 16 animals each. Four dosage levels of CCl_4 were utilized: 0, 1, 10, and 33 mg/kg po. The animals were dosed on a daily basis, 5 times weekly, for a total of 12 weeks. The body weight of each animal was recorded twice weekly. Blood samples were taken immediately prior to dosing at the following intervals: 2, 4, 6, 8, 10, and 12 weeks, and 2 weeks posttreatment. Five rats were randomly selected to serve as blood donors at the initial interval. These animals were lightly etherized and 2.5 ml of blood was withdrawn by needle puncture of the ventral caudal artery. Five different animals were selected for blood sampling at each of the next two sampling intervals. Thus each animal served twice as a blood donor, at 6-week intervals during the study. At the end of the 12-week dosing period, seven to nine rats per group were sacrificed. The remaining animals in each group were allowed to live for 13 days after cessation of dosing before being sacrificed. These rats were not gavaged with CCl_4 or corn oil during the recovery period.

Clinical chemistries and histopathology. At sacrifice, each animal was anesthetized with ether and the maximum amount of blood taken by cardiac puncture. The blood was refrigerated and allowed to coagulate. It was then centrifuged and the serum samples stored in a freezer at −20°C prior to analysis. Standard spectrophotometric procedures, involving monitoring the conversion of NADH to NAD over time, were used to measure sorbitol dehydrogenase (SDH) activity (Gerlach and Hiby, 1974) and glutamic-pyruvic transaminase (GPT) activity (Mattenheimer, 1971) in the serum. Ornithine-carbamyl transferase (OCT) activity in serum was measured by the technique of Drotman (1975), as modified by Kyle *et al.* (1983). Blood urea nitrogen (BUN) levels in serum were measured with a Gemini centrifugal analyzer.

After the blood samples were removed, the abdominal cavity of each animal was opened in order that tissue samples could be taken. In the subchronic study, the liver and kidneys were quickly removed and weighed. For all animals, a coronal slice of the left kidney and a 1-cm-wide strip of the left median lobe of the liver were taken and placed into buffered Formalin for fixation. The tissues were routinely processed into paraffin, cut at 2 μm, and stained with hematoxylin and eosin. The slides were coded and examined in a single blind fashion by a veterinary pathologist.

Statistical analyses. The statistical significance of differences between treated and control groups was assessed by paired or unpaired t tests, with $p < 0.05$ chosen as the minimum level of significance. The statistical significance

TABLE 1

CLINICAL CHEMISTRY DATA FROM FIRST ACUTE AND SUBACUTE STUDY[a,b]

Day of sacrifice after start of study	Daily dose of CCl_4 (mg/kg)	Total dose of CCl_4 (mg/kg)	OCT activity (nmol CO_2/ml)	SDH activity (IU/ml)	GPT activity (IU/ml)	BUN (mg/dl)
1	0	0	ND[c]	4 ± 0.7	12 ± 2	14.6 ± 0.8
	20	20	ND	4 ± 0.6	15 ± 2	19.0 ± 1.4
	40	40	ND	7 ± 1.8	20 ± 3	19.6 ± 1.0
	80	80	ND	9 ± 2.9	25 ± 4[d,e]	20.2 ± 1.1[d]
4	0	0	158 ± 62	4 ± 0.9	20 ± 5	19.8 ± 0.6
	20	80	163 ± 30	10 ± 3.9	26 ± 4	19.8 ± 0.4
	40	160	296 ± 25	51 ± 13.3[d]	55 ± 14[d]	21.0 ± 1.8
	80	320	260 ± 75	62 ± 3.0[d,e]	63 ± 16[d]	17.7 ± 1.1
11	0	0	92 ± 21	5 ± 0.2	20 ± 2	17.8 ± 1.0
	20	180	167 ± 17	78 ± 18.0[d]	44 ± 9[d]	19.0 ± 1.8
	40	360	372 ± 72[d]	155 ± 17.1[d]	351 ± 110[d]	22.2 ± 2.5
	80	720	737 ± 147[d,e]	241 ± 13.2[d,e]	469 ± 66[d,e]	24.6 ± 1.4[d]

[a] Rats of 300–350 g were dosed orally once daily for 5 days, rested for 2 days, and dosed once daily for 4 additional days.

[b] Values are expressed as mean ± SE for groups of five rats.

[c] ND—not determined.

[d] Significantly different from control at $p < 0.05$, paired t test.

[e] Significant increase with increasing dose at $p < 0.05$, analysis of variance.

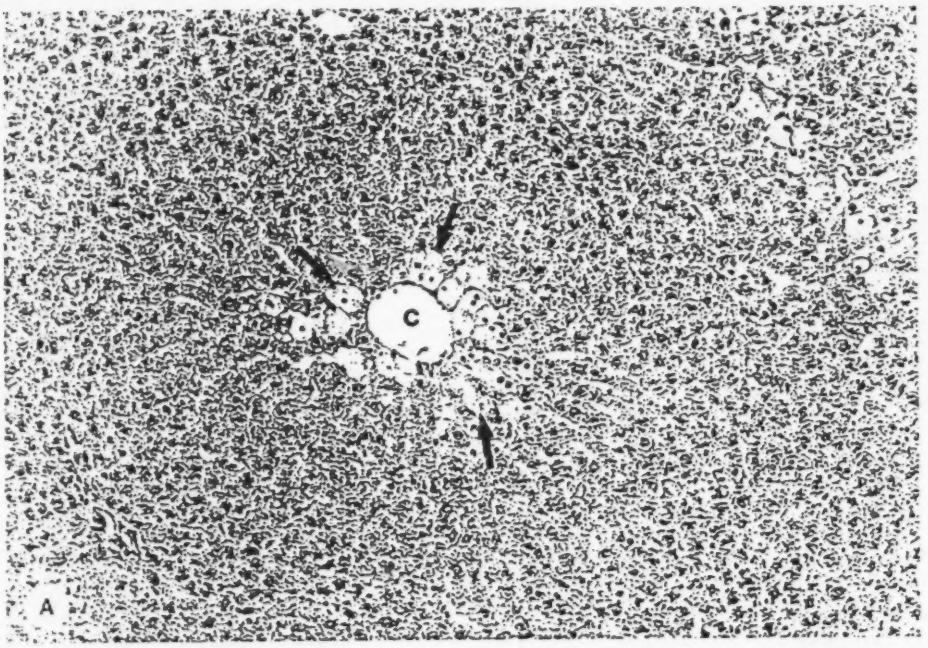
of changes in toxicity indices as a function of dose was determined by one-way analysis of variance.

RESULTS

First acute and subacute study. There was relatively little evidence of toxicity in 300- to 350-g rats 24 hr after a single dose of CCl_4. The 20- and 40-mg/kg doses did not produce significant alterations in any of the clinical chemistry indices (Table 1). Slight increases in serum enzyme levels and vacuolization of a few centrilobular cells in some liver lobules (Fig. 1A, Table 3) were observed in the 80-mg/kg animals. Their BUN value was significantly higher than the control value, but histopathological examination did not reveal any evidence of kidney damage in any group.

Liver damage after 4 consecutive days of CCl_4 ingestion was more pronounced than that

FIG. 1. (A) Liver lobule of a rat sacrificed 24 hr after oral administration of 80 mg CCl_4/kg. Change is limited primarily to vacuolization of hepatocytes (arrows) adjacent to the central vein (C). Hematoxylin and eosin, ×360. (B) Liver lobule of a rat sacrificed after ingestion of 80 mg CCl_4/kg for 4 consecutive days. Vacuolated cells are located primarily in the midzonal region (large arrows). Centrilobular hepatocytes have a lighter, more finely granular cytoplasm (small arrows) than do normal cells in the periphery of the lobule. Central vein (C). Hematoxylin and eosin, ×360. (C)Liver lobule of a rat dosed once daily with 40 mg CCl_4/kg po for 5 days, rested for 2 days, and dosed once daily for 4 additional days. The midzonal distribution of vacuolated hepatocytes gives the lesion a layered or laminated appearance. The position of the central vein is indicated by the large arrow, and the perimeter of the lobule outlined by small arrows. Hematoxylin and eosin, ×360. (D) Liver lobule of a rat dosed once daily with 20 mg CCl_4/kg po for 5 days, rested for 2 days, and dosed once daily for 4 additional days. Vacuolated hepatocytes are largely confined to the centrilobular area. Central vein (C). Hematoxylin and eosin. ×360.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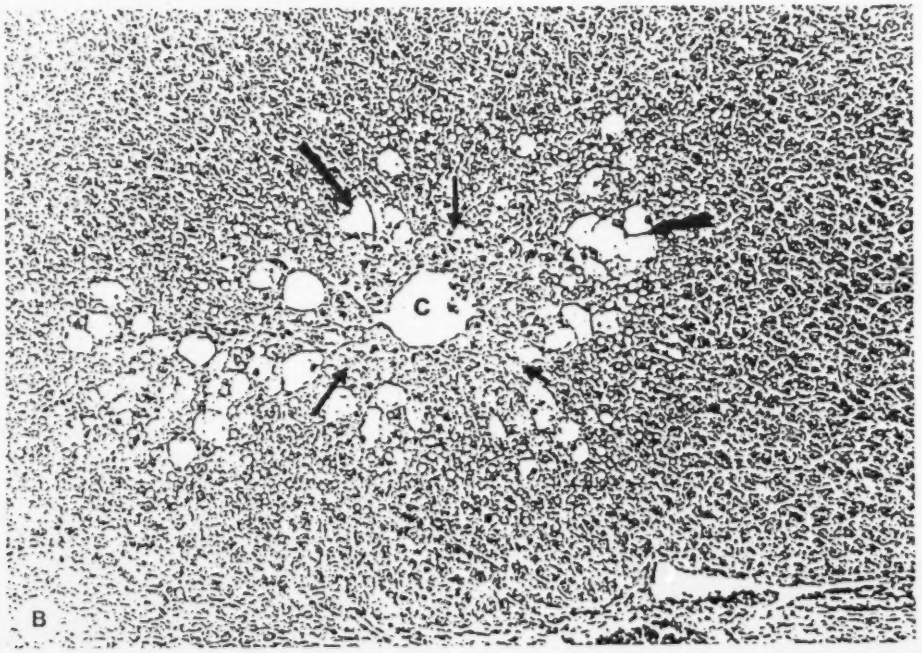
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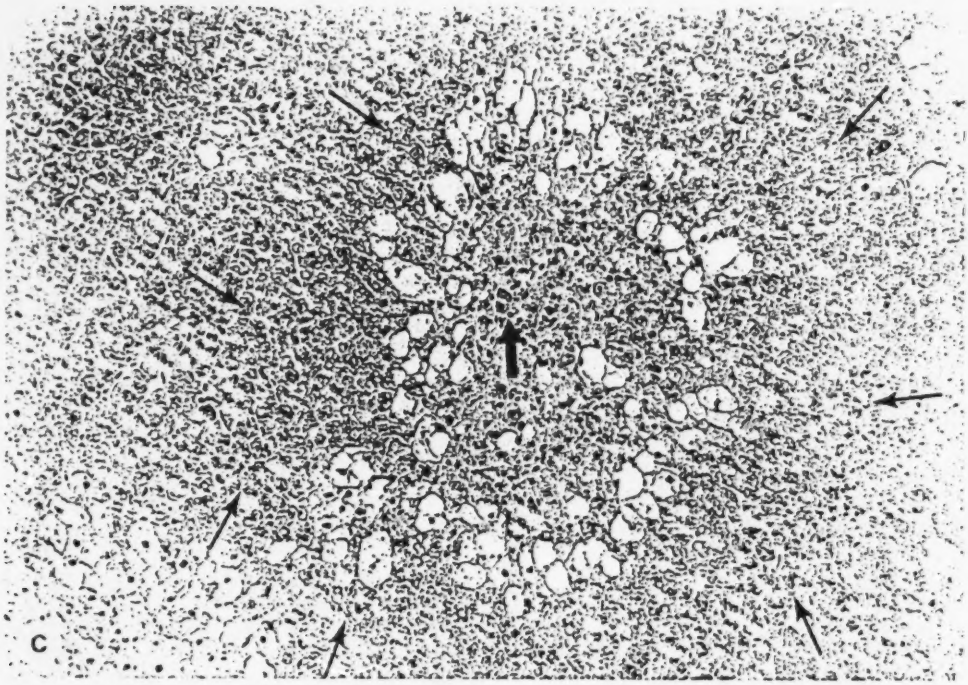
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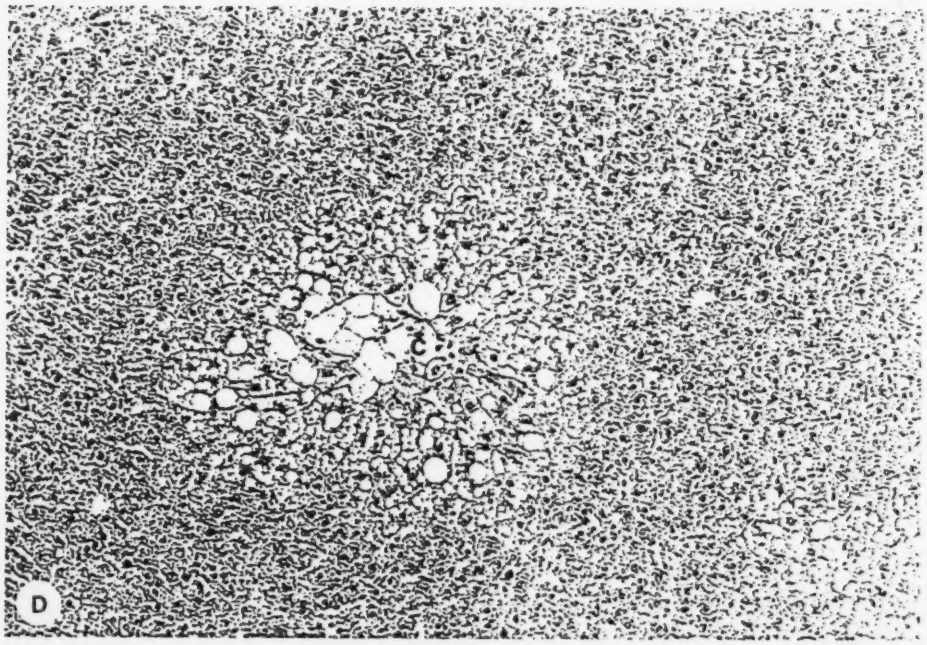
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seen at Day 1. Although the 20-mg/kg dose did not significantly alter any of the clinical chemistry indices (Table 1), it did cause vacuolization of hepatocytes adjacent to the central vein of most liver lobules in each animal. The 40- and 80-mg/kg doses produced comparable increases in SDH and GPT and vacuolization of 25 to 35% of the hepatocytes in each liver lobule (Fig. 1B, Table 3).

Dose-dependent increases in the clinical chemistry indices are manifest after 11 days of the regimen (Table 1). Ingestion of the lowest dose of CCl_4 (i.e., 20 mg/kg) results in elevated SDH and GPT levels and in prominent hepatic centrilobular vacuolization (Fig. 1D, Table 3). Ingestion of 40 and 80 mg/kg results in significant increases in serum levels of all three enzymes (Table 1). The BUN level in the 80-mg/kg animals is higher than that of the controls. Nevertheless, no chemically induced morphological alterations are visible in the kidneys of any animals.

An interesting sequence of histopathologic changes was seen over the 11-day study period. The initial hepatic lesion was ballooning vacuolization of centrilobular hepatocytes at 1 day in rats given 80 mg CCl_4/kg (Fig. 1A). At 4 days the cytoplasm of the centrilobular hepatocytes was finely granular and tinctorially lighter than that of normal hepatocytes, giving the cells a "ground-glass" appearance. These cells exhibited pleomorphic nuclei, with increased amounts of clumped chromatin. These abnormal hepatocytes were surrounded by a rim of ballooning, degenerating cells (Fig. 1B). Hepatocytes peripheral to the area of vacuolization appeared normal. At 11 days the centrilobular area of "ground-glass" cells was larger, and the thin rim of vacuolated cells was closer to the periphery of the lobule. This laminar lesion was apparent in the 40 mg/kg rats (Fig. 1C) and in some of the 20 mg/kg rats by 11 days. Necrotic cells were rarely seen at any dosage level during this study.

Second acute and subacute study. Toxicity manifestation 24 hr after a single administration of CCl_4 to 200–250-g rats varied with dose. The 20-mg/kg dose did not significantly alter any clinical chemistry parameter (Table 2) or cause histopathological changes (Table 3). Ingestion of 80 mg/kg resulted in a significant increase in SDH and GPT activities and in hepatic centrilobular vacuolization. The highest dose (i.e., 160 mg/kg) produced marked increaes in serum levels of each enzyme, as well as a significant decrease in BUN. These alterations were accompanied by hepatic centrilobular necrosis (Table 3).

The extent of liver injury after 4 consecutive days of CCl_4 administration is clearly dose dependent. Ingestion of 20 mg/kg results in small but statistically significant increases in SDH and GPT activities (Table 2). There is also vacuolization of hepatocytes adjacent to the central vein in liver lobules of the 20 mg/kg animals. Serum enzyme levels in both the 20- and 80-mg/kg animals are somewhat higher after 4 days than after just 1 day of dosing (Table 2). Conversely, the degree of effect in the 160-mg/kg group is less pronounced at 4 days than at Day 1. The hepatic centrilobular necrosis that was present in this group at Day 1 is no longer evident after 4 consecutive days of CCl_4 ingestion (Table 3).

There were statistically significant increases over controls in OCT, SDH, and GPT at all three dosage levels after 11 days of the exposure regimen. The increases in response to repeated administration of 80 and 160 mg CCl_4/kg were substantially larger than were increases at 4 days. It can be seen in Table 2 that there was pronounced intersubject variability at 11 days in these two high-dose groups. Substantial variability was also seen in the degree of hepatic necrosis in these animals (Table 3).

Histopathological changes in the liver paralleled clinical chemistry findings and were similar to changes seen in the first study. Again, the lesions were dose and time dependent (Table 3). Repeated administration of 20 mg/kg caused centrilobular vacuolization by 4 days and a laminar lesion by 11 days. The laminar lesions, when viewed in cross-section, consisted of centrilobular cells with a "ground-glass" appearance, a concentric rim of severely vacuolated cells, and a peripheral zone of normal cells. One dose of 80 mg/kg caused centrilobular vacuolization within 24 hr. Lesions

TABLE 2

CLINICAL CHEMISTRY DATA FROM SECOND ACUTE AND SUBACUTE STUDY[a,b]

Day of sacrifice after start of study	Daily dose of CCl_4 (mg/kg)	Total dose of CCl_4 (mg/kg)	OCT activity (nmol CO_2/ml)	SDH activity (IU/ml)	GPT activity (IU/ml)	BUN (mg/dl)
1	0	0	101 ± 39	4 ± 1.4	12 ± 5	17.8 ± 0.7
	20	20	24 ± 11	7 ± 0.4	16 ± 2	16.6 ± 0.9
	80	80	112 ± 18	43 ± 14.0[d]	40 ± 9[d]	15.4 ± 0.9
	160	160	7056 ± 3193[d]	204 ± 17.1[d,e]	385 ± 82[d,e]	9.2 ± 0.6[d]
4	0	0	78 ± 24	3 ± 0.4	22 ± 3	ND[c]
	20	80	98 ± 31	20 ± 3.5[d]	33 ± 2[d]	ND
	80	320	154 ± 10[d]	82 ± 15.3[d]	55 ± 6[d]	ND
	160	640	179 ± 50[d]	149 ± 44.8[d,e]	89 ± 6[d,e]	22.4 ± 1.9
11	0	0	68 ± 12	3 ± 0.2	10 ± 1	ND
	20	180	102 ± 24[d]	42 ± 5.0[d]	48 ± 7[d]	ND
	80	720	8345 ± 2610[d]	217 ± 50.3[d]	775 ± 245[d]	ND
	160	1440	588 ± 180[d]	251 ± 73.0[d,e]	1156 ± 429[d,e]	22.4 ± 1.0

[a] Rats of 200–250 g were dosed orally once daily for 5 days, rested for 2 days, and dosed once daily for 4 additional days.

[b] Values are expressed as mean ± SE for groups of five rats.

[c] ND—not determined.

[d] Significantly different from control at $p < 0.05$, paired t test.

[e] Significant increase with increasing dose at $p < 0.05$, analysis of variance.

TABLE 3

SEVERITY OF LIVER LESIONS IN THE ACUTE AND SUBACUTE STUDIES[a,b]

	Daily dose of CCl_4 (mg/kg)	Day of sacrifice after start of study	Extent of lesion[e]	Degree of necrosis[f]
First study[c]	20	1	0.0 ± 0.0	0.0 ± 0.0
		4	2.6 ± 0.0	0.0 ± 0.0
		11	3.5 ± 0.3	0.0 ± 0.0
	40	1	0.0 ± 0.0	0.0 ± 0.0
		4	4.0 ± 0.6	0.0 ± 0.0
		11	4.0 ± 0.0	0.0 ± 0.0
	80	1	1.6 ± 0.3	1.0 ± 0.2
		4	4.0 ± 0.6	0.0 ± 0.0
		11	5.6 ± 0.8	0.0 ± 0.0
Second study[d]	20	1	0.0 ± 0.0	0.0 ± 0.0
		4	1.4 ± 0.2	0.0 ± 0.0
		11	2.4 ± 0.2	0.0 ± 0.0
	80	1	2.0 ± 0.3	0.2 ± 0.2
		4	3.2 ± 0.2	0.0 ± 0.0
		11	5.4 ± 0.5	2.2 ± 0.4
	160	1	3.2 ± 0.2	3.0 ± 0.6
		4	4.4 ± 0.6	0.2 ± 0.2
		11	7.6 ± 0.2	1.8 ± 1.0

[a] Animals were dosed orally once daily for 5 days, rested for 2 days, and dosed once daily for 4 additional days.

[b] Values are expressed as mean ± SE for groups of five rats.

[c] Rats of 300–350 g were used.

[d] Rats of 200–250 g were used.

[e] Extent of lesions was graded on a scale of 0 (absent) to 8 (all of lobule affected).

[f] Degree of necrosis was graded on a scale of 0 (absent) to 8 (severe).

in the 80-mg/kg animals assumed the characteristic laminar shape by 4 days and progressed toward the lobular periphery by 11 days. The highest dose, 160 mg/kg, caused centrilobular vacuolization and necrosis at 24 hr (Table 3). At 4 days the lesions assumed an irregular laminar appearance, but necrosis was not seen. At 11 days the lesions did not have a laminar character. Instead, the majority of each lobule consisted of a mixture of vacuolated, necrotic, and pleomorphic hepatocytes. No chemically induced lesions were seen in the kidneys of animals at any dosage level.

Susceptibility to acute and subacute hepatotoxicity. CCl_4 was more hepatotoxic to 200- to 250-g rats than to 300- to 350-g rats. As two dosage levels (i.e., 20 and 80 mg/kg) were given in both of the foregoing studies, it is possible to compare the effects of CCl_4 on rats of two body weight ranges. As illustrated in Fig. 2, CCl_4 generally produced greater increases in SDH levels in the smaller rats. This was also true at most time points for GPT and OCT levels (data not graphed). Histopathological evaluation of livers of these animals also revealed that CCl_4 was more toxic to the smaller rats. Necrotic cells were very rarely seen in livers of the 300- to 350-g rats at any time point. In contrast, necrosis was observed at 11 days in each 200- to 250-g animal given 80 mg CCl_4/kg (Table 3).

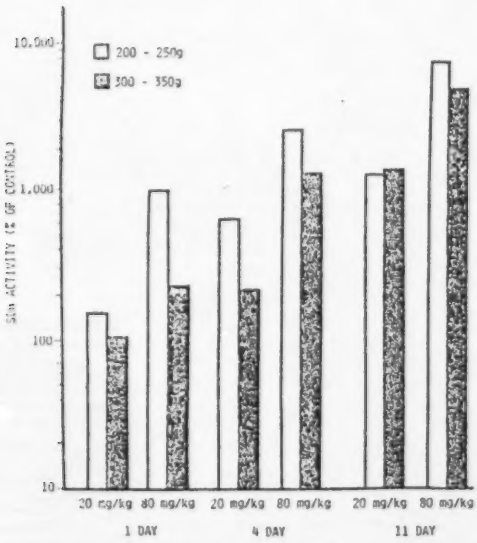


FIG. 2. Influence of body weight on CCl_4 susceptibility in acute and subacute studies. Groups of rats weighing 200–250 g and 300–350 g were dosed with 20 or 80 mg CCl_4/kg once daily for 5 days, rested for 2 days, and dosed once daily for 4 additional days. The animals were sacrificed after 1, 4, and 11 days of the regimens, and SDH activity measured. Bars represent mean values for groups of five rats.

Subchronic study. The influence of repeated CCl_4 ingestion over a 3-month period varied with dose. Oral administration of 1 mg/kg was without effect on clinical chemistry indices (Tables 4 and 5), liver and kidney morphology, body weight gain (Fig. 3), and liver:body weight and kidney:body weight ratios (data not shown). A dose of 10 mg/kg produced some modest changes, while 33 mg/kg caused marked changes.

Animals ingesting 10 mg CCl_4/kg exhibited slight alterations in some parameters. There were no significant changes in OCT or GPT during the course of the study, other than a small increase in GPT at the conclusion of the 12-week dosing period (Tables 4 and 5). SDH, the most sensitive clinical chemistry index, showed a two- to threefold increase over controls at most time points during the 3-month period. The degree of increase, however, was not always sufficient to be statistically significant. There was no evidence of progression of hepatic injury over the 12 weeks in the 10-mg/kg animals. Mild centrilobular vacuolization was seen in the liver of each animal examined at the 12-week sacrifice (Fig. 5A). There was no evidence, however, of necrosis, fibrosis, or other serious degenerative changes. There appeared to be a small increase over controls in liver:body weight ratio, but the increase was not statistically significant (data not shown). Kidney:body weight ratio (data not shown) and body weight gain (Fig. 3) were not affected by the 10-mg/kg dosage regimen.

Repeated oral administration of 33 mg CCl_4/kg resulted in substantial toxicity. SDH, OCT, and GPT were each considerably elevated at the initial evaluation (i.e., 2 weeks) and at each interval thereafter for the duration of the study (Tables 4 and 5). The extent of

TABLE 4

CLINICAL CHEMISTRY DATA FROM FIRST HALF OF THE SUBCHRONIC CCl_4 STUDY[a,b]

Week of evaluation after start of study	Daily dose of CCl_4 (mg/kg)	Total dose of CCl_4 (mg/kg)	OCT activity (nmol CO_2/ml)	SDH activity (IU/ml)	GPT activity (IU/ml)	BUN (mg/dl)
2	0	0	43 ± 17	2.9 ± 0.7	23 ± 1	ND[c]
	1	10	44 ± 13	8.3 ± 4.0	21 ± 1	ND
	10	100	31 ± 10	7.3 ± 1.5	25 ± 2	ND
	33	330	240 ± 56[d]	111.5 ± 17.0[d]	117 ± 28[d]	ND
4	0	0	21 ± 6	2.6 ± 0.1	22 ± 1	15.6 ± 1.5
	1	20	18 ± 4	ND	23 ± 2	17.8 ± 1.2
	10	200	26 ± 3	2.9 ± 0.3	22 ± 1	18.5 ± 1.2
	33	660	140 ± 29[d]	90.2 ± 14.7[d]	75 ± 21[d]	18.4 ± 0.5
6	0	0	55 ± 13	2.5 ± 0.5	10 ± 1	16.2 ± 0.2
	1	30	44 ± 5	2.4 ± 0.0	14 ± 2	15.4 ± 0.8
	10	300	40 ± 10	8.8 ± 0.8[d]	16 ± 2	15.4 ± 1.3
	33	990	251 ± 58[d]	119.3 ± 16.9[d]	82 ± 20[d]	15.0 ± 0.8

[a] Rats initially weighing 200–250 g were dosed orally on a daily basis, 5 times weekly, for a total of 12 weeks.
[b] Values are expressed as ± SE for group of five rats.
[c] ND—not determined.
[d] Significantly different from control at $p < 0.05$, paired t test.

TABLE 5

CLINICAL CHEMISTRY DATA FROM SECOND HALF OF THE SUBCHRONIC CCl_4 STUDY[a,b]

Week of evaluation after start of study	Daily dose of CCl_4 (mg/kg)	Total dose of CCl_4 (mg/kg)	OCT activity (nmol CO_2/ml)	SDH activity (IU/ml)	GPT activity (IU/ml)	BUN (mg/dl)
8	0	0	18 ± 3	2.2 ± 0.2	21 ± 1	ND[c]
	1	40	34 ± 9	1.6 ± 0.3	21 ± 1	ND
	10	400	30 ± 4	4.2 ± 1.5	19 ± 2	ND
	33	1320	151 ± 21[d]	110.6 ± 10.0[d]	130 ± 41[d]	ND
10	0	0	28 ± 8	3.5 ± 0.4	18 ± 1	14.8 ± 0.5
	1	50	23 ± 3	2.3 ± 0.6	20 ± 1	16.6 ± 1.0
	10	500	55 ± 10	7.6 ± 2.5[d]	23 ± 1	17.6 ± 0.5
	33	1650	148 ± 48[d]	134.8 ± 15.0[d]	617 ± 334	18.2 ± 2.5
12	0	0	45 ± 4	3.2 ± 0.4	20 ± 0.3	16.6 ± 0.7
	1	60	61 ± 12	1.9 ± 0.1	19 ± 1	17.1 ± 0.6
	10	600	69 ± 16	8.7 ± 2.0[d]	27 ± 2[d]	15.3 ± 1.2
	33	1980	247 ± 31[d]	145.7 ± 57.9[d]	502 ± 135[d]	16.2 ± 0.8
14	0	0	169 ± 33	4.7 ± 0.5	20 ± 2	15.1 ± 0.5
	1	60	111 ± 25	3.4 ± 0.5	20 ± 1	16.8 ± 0.3
	10	600	169 ± 42	4.3 ± 0.8	19 ± 1	16.7 ± 0.4
	33	1980	174 ± 24	6.0 ± 0.6	40 ± 7[d]	14.6 ± 2.0

[a] Rats were dosed orally on a daily basis, 5 times weekly, for a total of 12 weeks.
[b] Values are expressed as mean ± SE for groups of five to nine rats.
[c] ND—not determined.
[d] Significantly different from control at $p < 0.05$, paired or unpaired t test.

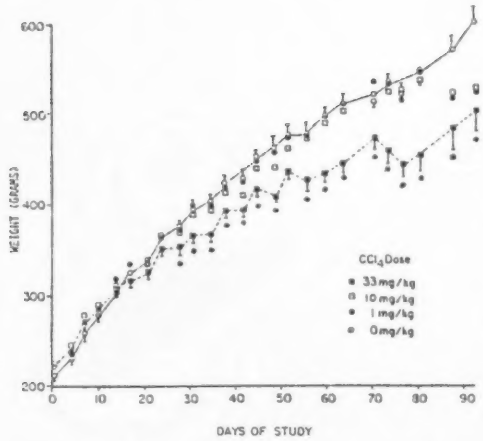


FIG. 3. Body weight gain in rats ingesting CCl_4 for 3 months. Brackets encase the mean and SD for groups of 7 to 16 rats. Solid line connects control values; broken line connects 33-mg/kg values. Asterisks indicate statistically significant difference between control and 33-mg/kg groups ($p < 0.05$), unpaired t test.

injury, as reflected by the three enzymes, remained relatively constant for the first 8 weeks in the 33-mg/kg animals (Fig. 4). There was an apparent rise in SDH levels at 10 and 12 weeks, though the increases over the 8-week value were not statistically significant. There was a pronounced rise in GPT at 10 and 12 weeks. Scrutiny of values of individual animals revealed that dramatic increases in two rats at each time point were largely responsible for the late increase in GPT activity. As shown in Fig. 4, the GPT and SDH values returned toward normal within 13 days after CCl_4 dosing was discontinued.

Only the 33-mg/kg dosage level significantly altered body weight gain and organ weights. A statistically significant decrease from controls in body weight gain was first manifest 30 days into the study (Fig. 3). This difference became more pronounced as the study progressed. There was a significant increase ($p < 0.05$) in the liver:body weight ratio of the 33-mg/kg rats at the 12-week sacrifice. Mean values ± SE for the control and the 33-mg/kg groups were 3.0 ± 0.3 and 4.4 ± 0.3, respectively. The kidney:body weight ratio in the treated group was also higher than the control, but the increase was not sufficient to be statistically significant (data not shown). These apparent increases in liver and kidney weight largely disappeared during the 13-day recovery period, although body weight remained significantly lower than in controls (Fig. 3).

The livers of the rats dosed with 33 mg CCl_4/kg exhibited extensive degenerative lesions at the 12-week sacrifice (Table 6). Vacuolization of hepatocytes was very common and most prominent in the periphery of lobules (Fig. 5B). Periportal fibrosis and bile duct hyperplasia (Fig. 5C) were present, though they varied in severity from one animal to another. Minimal fibrosis occurred around the central vein. Leukocyte infiltrates were commonly observed in the periportal areas. Although the

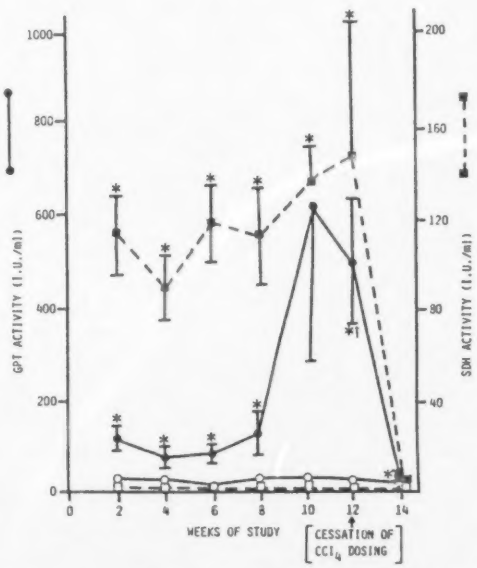


FIG. 4. Time course of GPT and SDH activities in the serum of rats ingesting 33 mg CCl_4/kg for 12 weeks. GPT (○) and SDH (□) activities in controls are also shown. Each point represents the mean ± SE for groups of five to nine rats. For sake of clarity, the small SE values of the controls are not shown. Asterisks indicate statistically significant difference between control and treated group, while † indicates significant differences between 8-week and designated group ($p < 0.05$), paired or unpaired t test.

TABLE 6

SEVERITY OF LIVER LESIONS IN THE SUBCHRONIC STUDY[a,b]

	Daily dose of CCl_4 (mg/kg)	Lipid vacuolization	Nuclear and cellular pleomorphism	Bile duct hyperplasia	Periportal fibrosis
12 weeks[c]	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	10	3.7 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	33	4.0 ± 0.4	5.7 ± 0.7	4.0 ± 0.6	3.7 ± 0.7
14 weeks[d]	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	1	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0
	10	2.9 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	33	1.4 ± 0.7	2.6 ± 0.7	3.4 ± 0.8	2.9 ± 0.8

[a] Rats initially weighing 200–250 g were dosed orally on a daily basis, 5 times weekly, for a total of 12 weeks.

[b] Extent of each lesion was graded on a scale of 0 (absent) to 8 (severe). Values are expressed as mean ± SE for groups of six to seven rats.

[c] Animals sacrificed after 12 weeks of the dosing regimen.

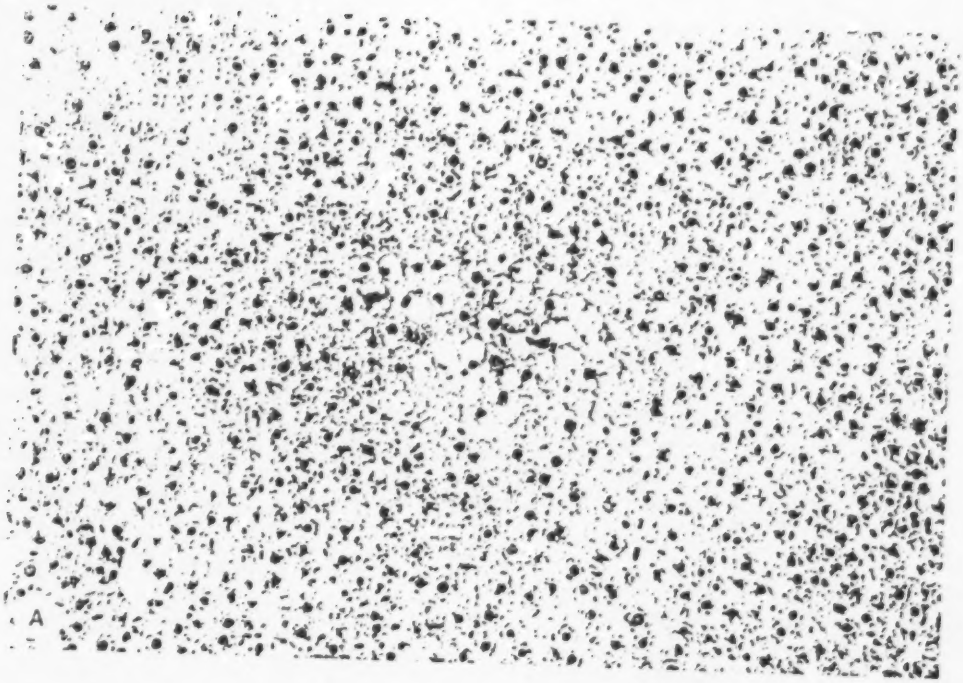
[d] Animals sacrificed 13 days after the end of the 12-week dosing regimen.

periportal fibrosis often made lobular boundaries quite distinct, pronounced hepatocellular pleomorphism distorted the lobular architecture. Hepatocytes with hyperchromatic nuclei were commonly seen, as were cells with multiple nuclei. Hyperplastic nodules were also found (Fig. 5C). Randomly distributed hepatocytes showed severe degenerative changes, including Councilman-like bodies, deeply eosinophilic cytoplasm, and pyknotic nuclei.

Hepatic lesions were still present in some groups of rats sacrificed 13 days after the last dose of CCl_4 (Table 6). There were no significant lesions in the 0 and 1 mg/kg groups. Four of the seven 10-mg/kg animals examined still showed mild centrilobular vacuolization. There were hepatic lesions in all seven of the 33-mg/kg animals, though the nature and severity of the lesions varied from animal to animal. Mild centrilobular vacuolization, for example, was present in just three of the seven animals. The severity of the fibrosis and bile duct hyperplasia was similar to that seen at the 12-week sacrifice. Pleomorphic hepatocytes were less prominent than at 12 weeks and were diffusely distributed throughout the liver lobules. Hyperplastic nodules were still found in some specimens (Fig. 5D).

There was no evidence of nephrotoxicity during the course of the subchronic study. No

FIG. 5. (A) Liver lobule of a rat dosed five times weekly for 12 weeks with 10 mg CCl_4/kg po. Vacuolated centrilobular hepatocytes occupy the center of the field. Hematoxylin and eosin, × 440. (B) Liver of a rat dosed five times weekly for 12 weeks with 33 mg CCl_4/kg po. Note that vacuolization is most pronounced in the periphery of the lobules. Ballooning cells and fibrous tissue (arrows) outline lobular boundaries. Hematoxylin and eosin, ×170. (C) Liver of a rat dosed five times weekly for 12 weeks with 33 mg CCl_4/kg po. Bile duct hyperplasia (B), fibrosis (small arrows), and a leukocyte infiltrate (large arrow) are present in the portal area. Note the variability in size and shape of the hepatocytes in the field. Hyperplastic nodule (N). Hematoxylin and eosin, ×440. (D) Liver of a rat dosed five times weekly for 12 weeks with 33 mg CCl_4/kg po, then allowed to recover for 13 days. A hyperplastic nodule (N) occupies the central portion of the field. The nuclei of cells in the nodule and surrounding tissue vary considerably in size and staining intensity. Note the presence of connective tissue (arrows). Hematoxylin and eosin, ×440.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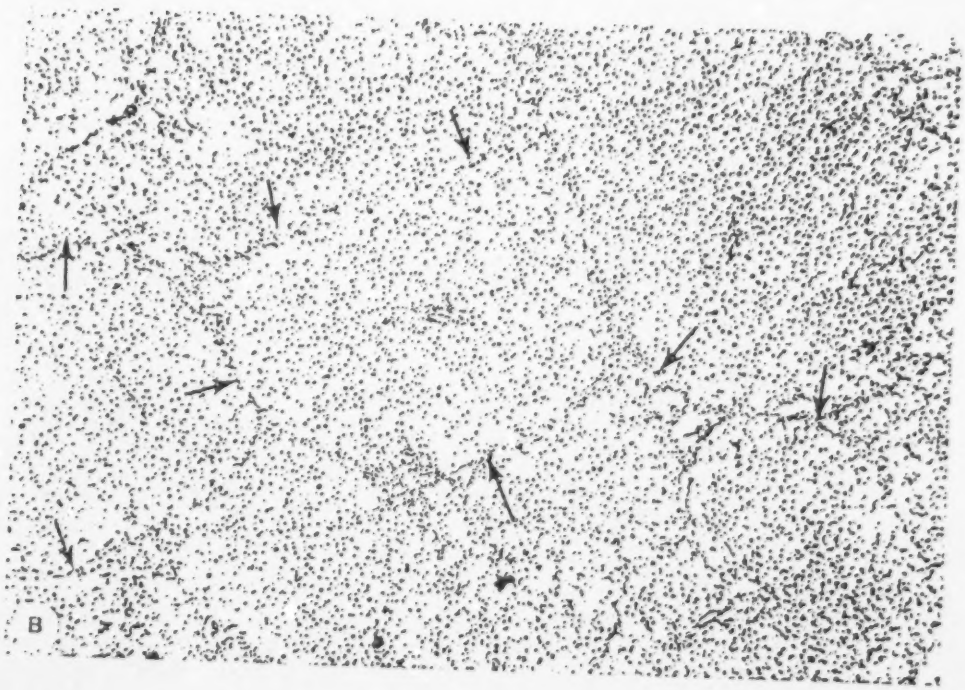
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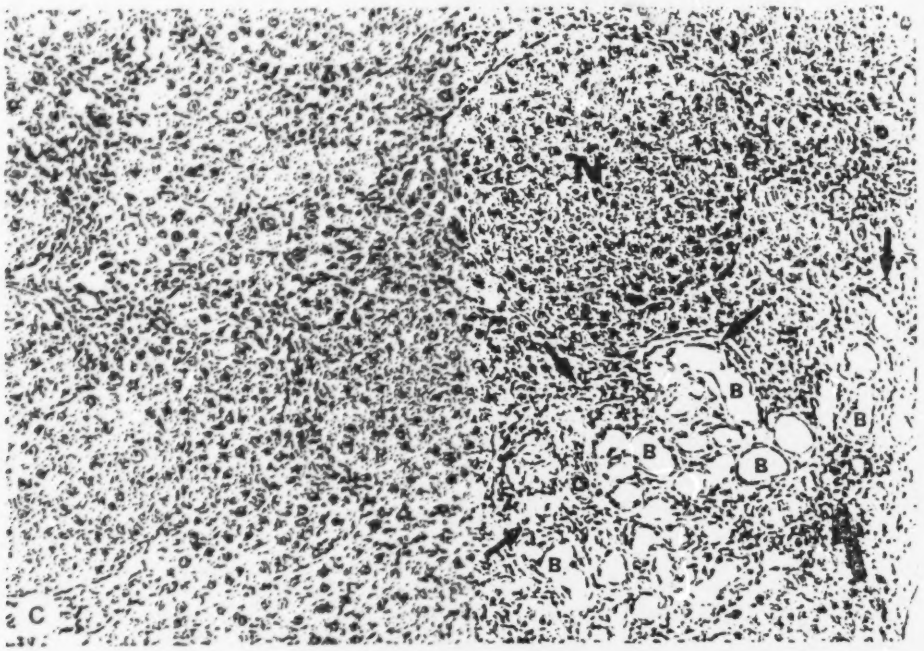
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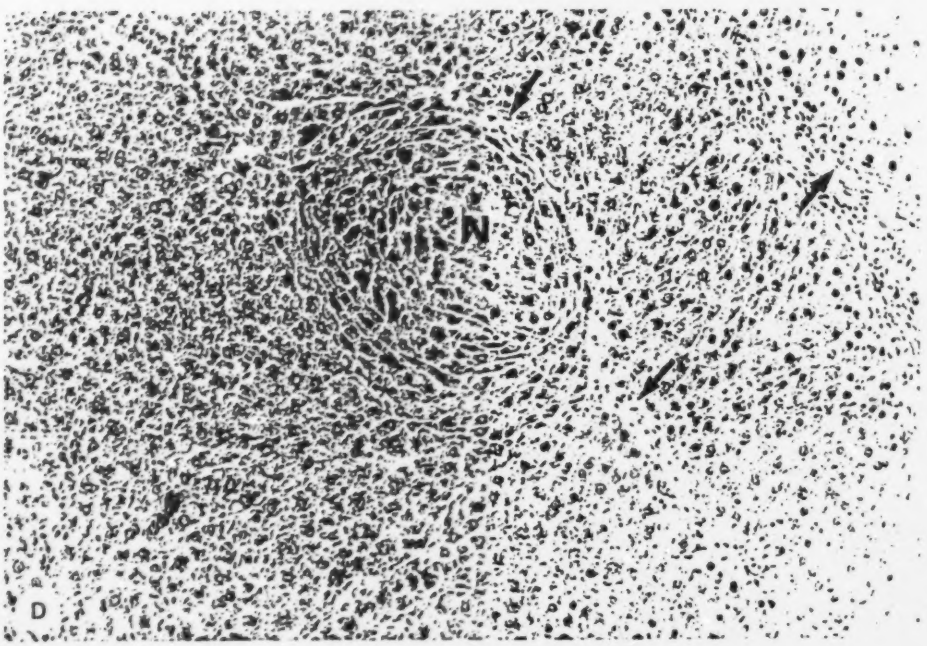
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significant alterations from controls were seen in BUN at any of the three dosage levels at any time point during the 12 weeks of CCl_4 administration (Tables 4 and 5). Although there appeared to be a slight increase in the kidney:body weight ratio in the 33-mg/kg group (data not shown), histopathologic changes were not found in the kidneys of this or the other dosage groups.

DISCUSSION

Findings in the present report should be useful in assessment of risks of short- and long-term CCl_4 ingestion. No adverse effects were seen in rats 24 hr after a single oral dose of 20 or 40 mg CCl_4/kg. Thus, 40 mg/kg can be considered the 1-day NOAEL under the conditions of our study. The 1-day LOAEL was 80 mg/kg, as it produced hepatic centrilobular vacuolization and elevated serum enzyme levels in both of the acute studies. The 1-day LOAEL may have been found to be 40 mg/kg, had this dose been utilized in the second acute study. The 200- to 250-g rats used in the second study proved to be more susceptible to CCl_4 hepatotoxicity than did the 300- to 350-g rats used in the first study. Although Korsrud *et al.* (1972) reported finding minimal morphological changes in centrilobular hepatocytes of rats dosed orally with 20 mg CCl_4/kg, 40 mg/kg would be a more realistic LOAEL in their study, as it produced modest but statistically significant changes in several indices of toxicity. It should be noted that their animals were fasted for 6 hr before dosing and for 18 hr afterward. Recknagel and Ghoshal (1966) saw a significant increase in liver triglycerides in fasted rats in response to a single oral dose of 16 mg CCl_4/kg. Reynolds and Yee (1968) also used fasted rats in a histochemical study of CCl_4 effects on the liver. A dosage of 4 mg/kg po caused a slight decrease in centrilobular glucose-6-phosphatase activity, while 40 mg/kg elicited a pronounced effect. These discrepancies in LOAELs can be attributed to the dissimilar parameters which were evaluated and to differences in experimental protocols. Fasting may be particularly important in this latter regard, as it is widely recognized that fasted rats are much more susceptible to CCl_4 hepatotoxicity (Diaz Gomez *et al.*, 1975b; Harris and Anders, 1980). Although our rats were not fasted, they were dosed soon after the beginning of their 12-hr dark/active cycle. Bruckner *et al.* (1984) have recently reported that nonfasted rats are more sensitive to CCl_4 hepatotoxicity during the initial part of their active cycle.

Age and body weight can have a profound influence on the toxicity of halocarbons. Although newborn rats are resistant to CCl_4 hepatotoxicity, rats 4 to 21 days old are reported to be as sensitive as adults (Dawkins, 1963; Cagen and Klaassen, 1979). Andersen and Jenkins (1977), however, find that the vulnerability of adult rats to 1,1-dichloroethylene hepatotoxicity decreases dramatically with increasing body weight. Thus it was of interest to us when conducting the two acute and subacute studies to see if larger rats were more resistant to CCl_4. This proved to be the case, as the 300- to 350-g animals rarely exhibited hepatic necrosis and generally had lower serum enzyme levels than their 200- to 250-g counterparts.

The rat appears to be of intermediate sensitivity to CCl_4 poisoning. Diaz Gomez *et al.* (1975a) report that CCl_4-induced hepatic necrosis and covalent binding decrease as follows: mouse > hamster $\geq$ guinea pig > rat > chicken. Klaassen and Plaa (1966, 1967) find that mice are 10 times more sensitive to acute liver injury by ip-injected CCl_4 than are dogs. Litchfield and Gartland (1974) observe no hepatic damage in dogs given 80 mg CCl_4/kg po. An oral dose of 320 mg/kg is required to clearly cause acute injury. The same investigators find no evidence of hepatotoxicity in dogs given 32 mg CCl_4/kg po daily for 8 weeks. In contrast, rats given 33 mg/kg po for 12 weeks in the current study experience serious liver damage, while a single oral dose of 80 mg/kg is sufficient to cause significant hepatic effects.

Attention is focused on the liver in the current study, as it is the most sensitive organ to CCl_4 in the rat and in most other species. Klaassen and Plaa (1966, 1967), for example, report that CCl_4 is the most hepatotoxic of a series of halocarbons tested in mice and dogs, but note that CCl_4 has little effect on the animals' kidneys. The lungs (Gould and Smuckler, 1971; Boyd *et al.*, 1980) and kidneys (Lundh, 1964; Striker *et al.*, 1968) of rats can be adversely affected by CCl_4, but very high acute doses are required. We see only slight occasional renal effects of questionable toxicological significance in the present investigation.

In consideration of the implications of repeated ingestion of relatively high doses of CCl_4 over a period of days, it is important to know whether a subject will experience progressively severe injury, or develop tolerance. The results of the two 11-day studies indicate that the outcome in the rat is influenced by dose. There is a progressive increase in enzyme levels and in severity of hepatic lesions over the 11-day period in the 20-, 40-, and 80-mg/kg groups. This is not the case for the 160-mg/kg group. Although a single 160-mg/kg dose produces marked elevations in serum enzyme levels and hepatic necrosis, these effects are much less pronounced after four daily doses. The development of tolerance to CCl_4 following pretreatment with the chemical is previously described by Dambrauskas and Cornish (1970) and by Ugazio *et al.* (1972). The phenomenon is attributed to the ability of the initial dose to depress the metabolic activation of subsequent doses. It is probable that 160 mg/kg was the only dose we utilized which caused serious enough hepatocellular injury to confer subsequent protection. Although toxicity is more pronounced at 11 days, it is possible there was still some degree of tolerance in the 160-mg/kg group, as enzyme levels and degree of necrosis are comparable at 11 days in the 80- and 160-mg/kg groups.

Histopathological examination reveals an interesting sequence of events within the liver lobule during the 11-day regimens that is not apparent when monitoring clinical chemistry parameters. On repeated CCl_4 exposure, the lesion progresses from the centrilobular area to the periphery of the lobule. With our highest doses, the expanding lesion eventually occupies the entire lobule and its laminated character is lost. The architecture of the lobules at 11 days is distorted, consisting of surviving cells with a "ground-glass" appearance, regenerating cells, vacuolated cells and scattered necrotic cells. These findings indicate that the cytotoxic effects of CCl_4 are reversible. Nevertheless, the appearance of prominent single cell necrosis at 11 days indicates that surviving hepatocytes' resistance can be exceeded by continued ingestion of high doses of CCl_4.

The ultrastructural changes which cause the "ground-glass" appearance of hepatocytes which survive injury are unknown. This term was coined to describe the appearance of hepatocytes in paraffin-embedded sections of livers from subjects with chronic viral hepatitis (Robbins, 1979). No one has apparently conducted an electron microscopic study of such cells in CCl_4-dosed animals. Stenger (1963) did carry out an ultrastructural study of hepatocytes in livers of rats dosed twice weekly for up to 35 weeks by sc injection of 0.2 ml CCl_4. This dose was apparently too high to produce a zone of "ground-glass" cells, as the report focused on ultrastructural changes in basophilic and lipid-laden hepatocytes. Such cells were seen in response to our highest subacute doses, where the laminated lesion with its central zone of "ground-glass" hepatocytes was no longer apparent.

The extent and nature of liver damage caused by subchronic CCl_4 ingestion varied with dose. The NOAEL was 1 mg/kg under the conditions of the study. The LOAEL was 10 mg/kg, as it elicited modest elevations in SDH levels and mild centrilobular vacuolization. As related previously, 33 mg/kg produced marked hepatotoxicity. Alumot *et al.* (1976) measured increased triglycerides and total lipids in livers of rats fed 20 to 25 mg CCl_4/kg daily in their diets for 5 to 6 weeks. Alumot *et al.* (1976) also conducted a chronic feeding

study in which male and female rats consumed an estimated 10 to 18 mg CCl_4/kg daily for up to 2 years. Although clinical chemistry indices revealed no signs of liver or kidney injury in survivors, most animals of each sex succumbed to chronic respiratory disease prior to the 2-year sacrifice. Male and female rats were given CCl_4 orally five times weekly for up to 78 weeks in doses ranging from 47 to 160 mg/kg (NCI, 1976). Bile duct proliferation, portal fibrosis, fatty degeneration, hepatocellular necrosis, and regenerative nodules were commonly seen in the livers of animals at all dosage levels. Fatty liver degeneration and cirrhosis were also seen in subchronic and chronic CCl_4 inhalation studies (Smyth *et al.*, 1936; Adams *et al.*, 1952; Prendergast *et al.*, 1967), but no attempt was made to estimate the actual systemic doses which produced these effects.

Histopathological evaluation of the rats we gave 33 mg CCl_4/kg po revealed certain degenerative changes in the liver which were not detectable by clinical chemistry tests. As described previously, periodic measurements showed serum enzyme levels to remain relatively stable over the 3-month dosing period, and to return toward normal within 2 weeks. Microscopic examination, however, revealed bile duct proliferation, fibrosis, lobular distortion, parenchymal regeneration and hyperplastic nodules, and single-cell necrosis. These degenerative changes associated with cirrhosis have been shown to cause marked disruption of the microvasculature and compromised perfusion of the tissue with blood (Hase, 1966; Popper and Udenfriend, 1970). Thus, the pathologic process itself can amplify the effects of the toxic agent.

Although the genesis of CCl_4-induced cirrhosis has been the subject of a number of detailed studies by other investigators, it is not possible to draw conclusions from these reports about the fibrogenic potency of ingested CCl_4. Stenger (1963) and Rubin *et al.* (1963), for example, typically give a single high dose of CCl_4 to rats once or twice weekly by sc injection. Such studies do extend our own findings, in that the previous studies are of longer duration and thus illustrate continued progression of the lesions. Rubin *et al.* (1963) report the fibrous septa connecting portal tracts and central veins to markedly increase in thickness and to join to surround hyperplastic nodules. CCl_4-induced septal fibrosis exhibits very slow spontaneous regression (Rabinovici and Wiener, 1961). Although cirrhosis and hyperplastic nodules are commonly seen in the livers of animals with hepatic tumors (Eschenbrenner and Miller, 1946; Della Porta *et al.*, 1961; Reuber and Glover, 1970), it is unclear whether a causal relationship exists.

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